Pilot study on HTR2A promoter polymorphism, -1438G/A (rs6311) and a nearby copy number variation showed association with onset and severity in early onset obsessive-compulsive disorder

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Abstract: A previous study showed that a single nucleotide polymorphism (SNP), -1438G/A (rs6311), found in the transcriptional control region of the gene that encodes the serotonin-receptor 2A (HTR2A) was associated with obsessive-compulsive disorder (OCD) in a sample of children and adolescents. In this study, we reanalyzed the association of this SNP with OCD in an enlarged population of 136 cases (55 previous + 81 new cases) and compared them to 106 newly recruited, healthy, age-matched controls. We also investigated whether this SNP or its copy number variations (CNV) was associated with OCD severity and age of onset. The CNV was analyzed in a DNA region located near rs6311. The results confirmed the association between the A-allele and early onset OCD in children and adolescents, with an odds ratio (OR) of 1.69 [95% CI (1.17, 2.46); p = 0.005]. Strikingly, we found that carriers of one copy (deletion) of the CNV were associated with a very early onset OCD (2.5 years earlier than the typical onset), and they had increased CY-BOCS scores (8.7 points higher compared to "normal" CNV and duplications); which is related to increased severity of OCD symptoms (p = 0.031; p = 0.004, respectively). Compared to the normal CNV and duplications, the association between the deletion and OCD showed an OR of 7.56 [95% CI (1.32, 142.84); p = 0.020]. These results pointed to the functional importance of this promoter region of HTR2A; it influenced the occurrence, the onset, and the severity of OCD.

DOI: https://doi.org/10.1007/s00702-011-0699-1

HTR2A –1438G/A is associated with early-onset obsessive-compulsive disorder and copy number variation affects onset and severity

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Running title: HTR2A polymorphism and CNV in early-onset OCD

Page No.: 21 Figures: 1 Tables: 2 Supplementary material: 0 References: 59

Abstract word count: 235 Introduction word count: 684 Total word count: 3271

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Abstract

A previous study showed that a single nucleotide polymorphism (SNP), –1438G/A (rs6311), found in the transcriptional control region of the gene that encodes the serotonin-receptor 2A (HTR2A) was associated with obsessive-compulsive disorder (OCD) in a sample of children and adolescents. In this study, we reanalyzed the association of this SNP with OCD in an enlarged population of 136 cases (55 previous + 81 new cases) and compared them to 106 newly recruited, healthy, age-matched controls. We also investigated whether this SNP or its copy number variations (CNV) was associated with OCD severity and age of onset. The CNV was analyzed in a DNA region located near rs6311. The results confirmed the association between the A-allele and early-onset OCD in children and adolescents, with an Odds Ratio (OR) of 1.69 (95% CI: [1.17, 2.46]; p=0.005). Strikingly, we found that carriers of one copy (deletion) of the CNV were associated with a very early-onset OCD (2.5 years earlier than the typical onset), and they had increased Y-BOCS scores (8.7 points higher compared to "normal" CNV and duplications); which is related to increased severity of OCD symptoms (p=0.031; p=0.004, respectively). Compared to the normal CNV and duplications, the association between the deletion and OCD showed an OR of 7.56 (95% CI: [1.32, 142.84]; p=0.020). These results pointed to the functional importance of this promoter region of HTR2A; it influenced the occurrence, the onset, and the severity of OCD.

Keywords: obsessive-compulsive disorder; HTR2A polymorphism; child and adolescent psychiatry; copy number variation

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Introduction

Obsessive-compulsive disorder (OCD) is a heterogeneous psychiatric disorder characterized by clinically significant, recurrent, intrusive, and disturbing thoughts (obsessions), accompanied by repetitive, stereotypic behaviors, usually associated with anxiety or dread (compulsions) (1). According to the National Comorbidity Survey Replication, the median age of OCD onset is 19 years. However, symptoms of OCD often begin during childhood or adolescence; in fact, 21% of all cases start experiencing symptoms by 10 years old (2, 3). Kessler and colleagues found that, among anxiety disorders, OCD had the highest percentage (50%) of severe cases (3); furthermore, early-onset OCD often develops a chronic course and has a poor long-term outcome. Formal genetic studies showed that OCD had a high familial component, particularly early-onset OCD (4); however, the definition of early-onset OCD differs among studies. Dysfunction of the serotonin (5-HT) system has been implicated in OCD and is thought to contribute to behavioral traits, like perfectionism, obsessiveness, anxiety, and depression (5-7). Additionally, there is evidence that 5-HT is involved in eating disorders (8, 9). Obsessive, restrictive behaviors and perfection-seeking personalities are often found in patients with anorexia nervosa (9, 10). Selective serotonin re-uptake inhibitors (SSRI; e.g., fluoxetine) that block the serotonin transporter represent the most effective pharmacological treatment of OCD, early-onset OCD, and anxiety disorders. A meta-analysis (11) was conducted on 12 randomized double-blind placebo controlled studies that tested SSRI and clomipramine treatments for OCD and included 1044 affected children and adolescents. The effect size was a pooled standardized mean difference of 0.46 for SSRIs and clomipramine compared to placebo (11). In the pediatric OCD Treatment Study (POTS), fluoxetine was found to be effective, and it ameliorated the effects of behavioral therapy (12). Therefore, most genetic association studies in OCD have investigated genes related to serotonergic signaling. Association studies in early-onset OCD are rare, but there is extensive evidence that the heritability of early-onset OCD is higher than the

heritability of later onset OCD (onset after 18 years old) (4). Our group previously conducted an association study to determine whether early-onset OCD was associated with the single nucleotide polymorphism (SNP), -1438A/G (rs6311), in the serotonin receptor 2A (HTR2A) promoter (13). We applied a case-control design in a pediatric sample and detected a significant association of the A-allele with OCD (13). Our results were in accordance with the results from a study conducted by Enoch and colleagues in adult patients with OCD (10, 14). Those authors also described a correlation between the -1438A-allele and early-onset OCD that was determined retrospectively (10, 14). In those studies, the effect size was more pronounced in female patients (14). However, several other case-control association studies on adult OCD subjects (15-17) reported no association with SNP rs6311. Similarly, several association studies for the synonymous T102C (rs6313) variant, which was reported to be in complete linkage disequilibrium with rs6311 in different populations (18-21), reported conflicting results in adult OCD subjects (16, 17, 19, 22-24). Dickel et al. (2007) (25) conducted transmission-disequilibrium tests and failed to find an association between the rs6311 polymorphism and early-onset OCD (25); however, they reported a significant association between OCD and a comorbid tic disorder. Therefore, further studies are required to resolve these conflicting results.

In parallel, recent investigations have implicated *de novo* and/or rare copy number variations (CNVs) as potentially pathogenic factors in attention-deficit/hyperactivity disorder (26-28), autism (29-31), and schizophrenia (30, 32, 33). The duplication or deletion process of CNVs can disrupt a variable number of genes, which can result in alternate gene products or changes in gene dosage. Moreover, the disruption of regulatory regions in the genome can lead to altered gene expression, and this may contribute to disease predisposition. Given the highly heritable and variable nature of OCD, we hypothesized that individual, rare CNVs near the SNP rs6311 might contribute to OCD risk and severity.

In the present study, we extended the number of patients included in our previous study (13) and

also included new independent controls. We aimed to confirm our previous results of the association between the rs6311 polymorphism and early-onset OCD. In addition, we aimed to investigate the association between early-onset OCD and CNVs located near SNP rs6311.

Results

Demographic data

The study population included slightly more males than females in both the OCD and the healthy control groups (p=0.51). The healthy controls were significantly younger than the OCD cases (p<0.0001); however, the average age of the controls was not significantly different from the average age of the subjects at OCD onset (p=0.21).

HTR2A -1438 A/G polymorphism and CNV associations with age of onset and severity of OCD

The HTR2A genotype was not significantly associated with age of onset or severity of OCD in the case population (Table 1A). In addition, there was no significant difference between "normal" and "high" CNV with respect to age of onset or severity (t-test p=0.47 and p=0.30, respectively). Because we hypothesized that the deletion ("low" CNV) would affect the phenotype by impairing the function of the promoter activity, we pooled the normal and high data into a joint normal/high CNV group for comparisons to the low CNV group (Table 1B). This analysis method revealed a significant association between "low" CNV and the age of onset (p=0.031); the estimated average age of onset was 2.5 years earlier than that of the normal/high group (95% confidence interval (CI): [0.2 years, 4.8 years]). Similarly, we observed a significant association between "low" CNV and the OCD severity measured with Y-BOCS scores (p=0.004); the estimated average severity score was 8.7 points higher than that of the normal/high group (95% CI: [2.9 points, 14.5 points]).

Case-control analysis of the HTR2A -1438 A/G polymorphism and CNV

The association between SNP rs6311 and OCD was first analyzed in only the 81 newly recruited

patients with OCD (those not included in the previous study (13). There was a significant difference between the genotype distributions of controls and these OCD cases (p=0.030). Note that the previous study, with a different control group and a smaller OCD group, also reported a significant association between genotype and OCD (p=0.046). When all 135 available cases with SNP information were included, the association was highly significant (p=0.007). We also found that the OCD cases showed a higher frequency of the AA genotype (19.3%) compared to the healthy controls (16%) (Table 2). The estimated OR for the A allele was 1.69 (95% CI: [1.17, 2.46]; p=0.005), adjusted for CNV. Assuming an additive effect of the A allele, this indicated that the risk of OCD was increased by 69% in patients with the heterozygous GA genotype and by 286% for the homozygous AA genotype.

The association (adjusted for SNP) between the HTR2A CNV ("low" vs. "normal/high") and OCD was also significant (p=0.020). The SNP-adjusted OR estimated for a "low" CNV relative to a "normal/high" CNV was 7.56 (95% CI: [1.32, 142.84]). We did not find a significant gender effect in our case-control analysis (p=0.72).

Discussion

In this study, we confirmed the previously described association between the A-allele of SNP rs6311 and OCD, particularly in children and adolescents (10, 13, 14). We estimated that the OR per A-allele was 1.69 (95% CI: [1.17, 2.46]) for early-onset OCD, and we found no significant association between age of onset and OCD severity. This might be due to the fact that the OCD population in this study contained only patients with very early-onset of the disease (average age of onset was 11.1±3.2 years, range 3-17.4 years). Thus, we could not rule out an age dependent association for the-rs6311 A-allele. For example, age might be detected as a factor in an analysis of patients that experienced OCD onsets at all ages, from 3 to >18 years. That association could be a result of polymorphism influence on HTR2A gene expression through the promoter activity.

An extensive reporter gene assay suggested that the rs6311 SNP modulated HTR2A promoter activity (34); however, another study on the effect of the rs6311 SNP on promoter activity revealed no significant difference in expression of HTR2A mRNA (18).

An important feature of HTR2A expression is its monoallelic expression. HTR2A is imprinted in human fibroblasts, which express only the maternal allele (35). Polymorphic imprinting in adult human brains was reported in a subset of heterozygotes, but the parental origin of the expressed allele was not analyzed (36). Analyses of DNA and mRNA related to the 102T/C polymorphism (rs6313) of the *HTR2A* gene revealed that 102C was expressed at lower levels than 102T in brain tissue (37). In contrast to this biallelic expression observed in the brain, monoallelic expression of HTR2A was common in peripheral lymphocytes (37). However, a familial study revealed that imprinting was not responsible for the monoallelic expression in peripheral lymphocytes (37). A recent report demonstrated that HTR2A mRNA expression in human fibroblast cell lines reflected a significant overall genotypic effect; subjects with the AA genotype at the –1438 locus (rs6311) had significantly higher levels of HTR2A mRNA compared to subjects with the AG genotype, but not compared to those with the GG genotype (38). Moreover, the –783A/G (rs6312) polymorphism significantly modified the effect of –1438A/G on HTR2A mRNA expression. When the -783 G-allele occurred in conjunction with the G-allele of the rs6311 polymorphism, the HTR2A promoter activity was significantly reduced compared to other genotypes (38). A recent study reported an *in silico* selection of phenotypes that reflected different polymorphisms in the HTR2A gene (39). The SNP rs6311 was found to cause loss of the E2F1 transcription factor site and the gain of two other transcription factor sites (HAND1E47 and SMAD; See Fig. 1). Another investigation of epigenetic factors affected by rs6311 revealed that a newly created E47 binding site (A-allele), a glucocorticoid receptor binding site at position -1420, and an Sp1 binding site at CpG methylation site +1224 caused alterations in methlylation rates at both -1420 and -1439 sites (40). Therefore, modifiers should be taken into account in

functional studies.

One problem with genetic analyses is the inability to completely match a control group or de-emphasize ethnic differences between patients and controls. In the present study, we included 136 German patients with early-onset OCD, of which 107 patients were children from Würzburg, Germany. All patients and controls were of German origin. Furthermore, all 106 healthy German children were also recruited at the Department of Child and Adolescent Psychiatry of the University of Würzburg; therefore, we assumed that we were able to de-emphasize ethnic stratification.

We also found that one copy (deletion) of a CNV located near the rs6311 was associated with very-early-onset OCD and an increased Y-BOCS score, which was related to enhanced symptom severity. Of note, the frequency of this deletion in our OCD group (n=8) was increased compared to that in the healthy controls (n=1). Characterizations of CNVs and their associated polymorphisms are important, because they may provide key links to understanding of the genetic basis of quantitative traits and the different susceptibilities to psychiatric diseases. An initial study that explored the effects of CNVs on gene expression in lymphoblastoid cell lines from human populations reported that changes in copy number explained nearly 20% of the detected gene expression variation (41). This was suggested to result from altered dosages of genes that mapped within CNVs; however, it may also potentially result from the impact of CNVs on neighboring genes (41, 42). Recent studies have shown that CNVs can influence the expression of genes both within the CNV and outside the CNV, in the vicinity of up to half a megabase (43). It was suggested that, for disorders like Williams-Beuren (WBS; MIM 194050), Prader-Willi (MIM 176270), Angelman (MIM 105830), and DiGeorge/velocardio-facial syndromes (DG/VCFS; MIM 188400), patients with duplications might have different clinical syndromes and milder phenotypic features than those with deletions. This might be explained by the fact that excess information tends to be less detrimental to the organism than a deficiency

(44, 45). This effect might explain our observations of the effect that the "low" CNV had on the HTR2A promoter region; this effect might also influence promoter activity. A potential mechanism might be the loss of transcription factor sites within the CNV deletion (see Fig. 1). However, this theory requires further investigation.

These findings may suggest additional applications for investigating the genomic effects associated with OCD in large-scale populations.

Material and Methods

Subjects

The study sample comprised patients that had received in-patient treatment at the Department of Child and Adolescent Psychiatry of the Universities of Würzburg, Marburg, Aachen, and Freiburg. All patients and healthy controls were of German ethnicity. All participants and the parents of minors gave written informed consent. The ethics committees of all participating universities approved the study.

Patients fulfilled the diagnostic criteria for current OCD according to the Diagnostic and Statistical Manual of Mental Disorders, 4th edition (DSM-IV) ((1) and the International Statistical Classification of Diseases and Related Health Problems, 10th Revision (ICD-10) (46). To assess the criteria for OCD and comorbid psychiatric disorders, both patients and parents were interviewed separately with the semi-structured diagnostic interview of psychiatric disorders in children and adolescent (DIPS; children and parents version) (47). The DIPS is used to assess a wide range of psychiatric disorders in childhood and adolescence, including schizophrenia and allied disorders, autistic spectrum disorders, attention-deficit/hyperactivity disorder (ADHD), and conduct and oppositional-, anxiety-, affective-, eating- and tic-disorders, diagnosed according to the criteria of the ICD-10 (46). The DIPS further includes a general clinical screening component to assess substance use, abuse, and somatic diseases.

The children and adolescents were interviewed and evaluated with the Yale-Brown Obsessive Compulsive Scale (Y-BOCS) (48) to assess further characteristics and severity of OCD. A summary score above 16 points was determined to be the cut-off for clinical impairment caused by OCD symptomatology; a score of 38 points was the maximum for describing severity according to the Y-BOCS. Of note, the DSM-IV does not require children to fulfill the criterion of insight regarding the irrationality of the symptoms. This may result in low summary scores; therefore, it is possible that the Y-BOCS evaluations underestimated the true severity of symptomatology. For diagnostic assessments of present and lifetime Tourette´s Syndrome and tic-disorders, we used the adapted German version (49) of the Child and Adult Schedule for Tourette and Other Behavioral Syndromes (STOBS), according to Pauls et al. (50).

All interviews were performed by senior clinicians of the university clinics for child and adolescent psychiatry. Subjects with comorbid disorders were only included in the study when the OCD symptoms were most prominent, based on two criteria: 1) OCD had to predate the onset of the comorbid disorders (except ADHD), and 2) two senior clinicians independently diagnosed the predominance of the OCD symptoms.

Exclusion criteria for patients were: Lifetime history of psychotic disorders, Gilles-de-la-Tourette's syndrome and chronic tic-disorder, autistic spectrum disorder (e.g., infantile autism, atypical autism, Asperger´s syndrome), alcohol dependence, or mental retardation (IQ < 70).

The patient sample consisted of 136 patients (children and adolescents, 77 males, 59 females) with a mean age at investigation of 13±2.9 years (range: 4.7 to 17.9 years). The mean age of onset was 11.1±3.2 years (range: 3 to 17.4 years). The patient sample was a superset that comprised 55 children with OCD from a previous study (13), plus 81 newly recruited children with OCD. Of the total 136 patients, 74 (54.4%) had no comorbid diagnosis, and 61 had a current diagnosis of comorbidity. The most frequent comorbidities were ADHD (n =15), anxiety disorders (n =10), depressive disorders (n =8), eating disorders (n =5), and conduct disorders (n

=4). The remaining cases had one of the following disorders: dyslexia, enuresis, or other behavioral and emotional disorders. Thirteen patients had a lifetime-diagnosis of tic-disorder according to STOBS.

In a previous case-control study regarding the −1438G/A polymorphism in early-onset OCD (13), the control group consisted of students that were older than the patients. For this study, we recruited 106 healthy German children from the Department of Child and Adolescent Psychiatry of the University of Würzburg to use as new independent controls. The mean age of the controls was 11.6±2.6 years, and 38.7% of the controls were female. They were screened with the Child Behavior Checklist (CBCL) (51) and a validated German depression self-rating inventory (DIKJ) (52). All controls and their mothers were interviewed with the Schedule for Affective Disorders and Schizophrenia for school-aged children (Kiddie-Sads) (53). The intelligence level was assessed with the Culture Fair Test (CFT 1/20) (54, 55). Exclusion criteria for participation of controls were: any severe somatic or neurological disease; any psychiatric diagnosis according to the Kiddie-Sads; a DIKJ score that exceeded the threshold of 18 for clinically relevant depressive symptoms; any T-score above 63 on the internalizing, externalizing, or total score of the CBCL; or an IQ below 80.

Genotyping for the HTR2A -1438 A/G polymorphism

Genomic DNA was extracted from whole blood with the desalting Proteinase K methodology (56). Genotyping for the -1438 A/G polymorphism (rs6311) (location 47,471,478 on the GRCh37; 13q14 q21) was conducted with the TaqMan® SNP genotyping assay (Assay no. C-8695278-10; Applied Biosystems Co., Germany) on the iCycler iQ Real-Time thermocycler (BioRad Laboratories Inc., Germany), according to manufacturer instructions. Genotypes were assessed according to the Allelic discrimination method with the iCycler IQ software (version 3.1.7050; BioRad Laboratories, Germany).

Copy number variation analysis

The CNV located 68 bp downstream of SNP rs6311 (location 47,471,310-47,471,410 on GRCh37; 13q14.2a; amplicon size 100 bp; Fig. 1) was analyzed with the TaqMan® Copy Number Assay (Assay no. Hs06366812_cn; Applied Biosystems Co., Germany) on the iCycler iQ Real-Time thermocycler (BioRad Laboratories Inc., Germany), according to manufacturer instructions. Each sample was adjusted to 20 ng genomic DNA per well. The TaqMan® Copy Number Reference assay for RNase P was used for normalization. Four replicates were run per sample. The CNV per sample were evaluated by the Cycles threshold (C_T) values assigned with the CopyCaller® software (version 1.0; Applied Biosystems Co., Germany). A "normal" CNV was defined as two copies; a "low" CNV was defined as ≤1 copy (deletion); and a "high" CNV was defined as ≥3 copies (duplication). For each sample, an independent replication of the assay was conducted in order to eliminate false determinations of CNV.

Statistical Analysis

All statistical tests were two-sided with an alpha-level of 0.05. The results were unadjusted for multiple testing, because the analysis was exploratory and the number of tests was comparably small. All confidence intervals were 95% profile likelihood intervals (57).

Gender and age distributions were compared with Fisher's exact test and the Student t-test, respectively. Differences in average OCD onset and severity (measured by the Y-BOCS score) were assessed with one- and two-way ANOVAs for separate and simultaneous comparisons, respectively. In the case-control analysis, discrepancies in SNP and CNV distributions were separately tested with Armitage's trend test and Fisher's exact test, respectively (58). Logistic regression for a simultaneous association analysis of SNP and CNV with OCD assumed an additive model for SNP and an unrestricted model for CNV. An additional interaction of SNP with gender was tested with the Student t-test.

The computations were performed with the statistical computing software R (59), version 2.11.0.

Acknowledgements

We would like to express our gratitude to all participants. In addition, we would also like to thank M. Hofmann, T. Elpel, and G. Ortega for their excellent technical assistance. The study was supported by the Deutsche Forschungsgemeinschaft WA 1618 and the KFO 125.

Conflicts of interest

The authors declare no conflict of interest.

Author's contribution

- Designed research- Susanne Walitza, Edna Grünblatt
- Performed research- Susanne Walitza, Edna Grünblatt
- Contributed new reagents or analytic tools- Susanne, Walitza, Marcel Romanos, Tobias Renner, Michael Simons, Christoph Wewetzer, Christian Fleischhaker, Helmut Remschmidt, Andreas Warnke
- Analyzed data- Daniel Sabanés Bové, Leonhard Held

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Figure Legend

Figure 1. Alignment of CNV and SNP rs6311 on the HTR2A promoter sequence and their effects on transcription factors. A) The alignment of HTR2A on chromosome 13. B) The HTR2A gene alignment with its promoter region (not to scale). C) The promoter region with the CNV and rs6311 alignment (not to scale); the indicated transcription factors may be affected according to PROMO version3 (http://algenn-lsi.ups.es). Red: G-allele; Green: A-allele; +: gain, and –: loss, of transcription factors (according to Pita et al. 2010).

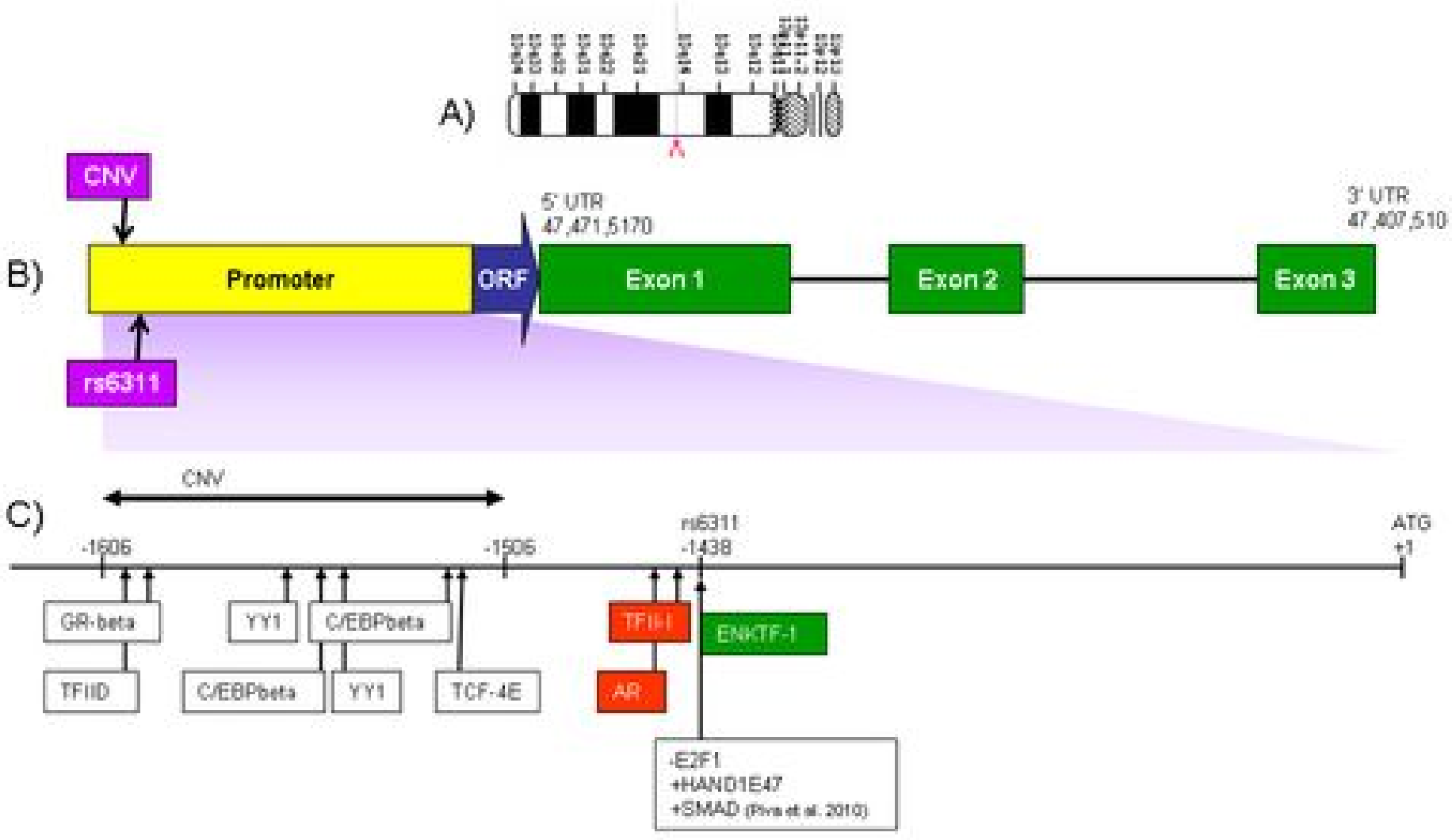
A)
B)
CNV
Promoter
ORF
5' UTR
47,471,5170
Exon 1
Exon 2
Exon 3
3' UTR
47,407,510
rs6311
C)
CNV
-1606
-1505
rs6311
-1438
ATG
+1
GR-beta
YY1
C/EBPbeta
TFIID
C/EBPbeta
YY1
TCF-4E
TFII-I
ENKTF-1
AR
-E2F1
+HAND1E47
+SMAD (Piva et al. 2010)

Table 1: Age at onset and severity of OCD, based on the SNP rs6311 genotype (A) and the CNV size (B)

A) Genotype (rs6311)	**Age at onset (years) (mean±SD)**	**N**	**Missing**	**Y-BOCS (score) (mean±SD)**	**N**	**Missing**
GG	11.0±3.3	38	1	23.9±8.4	34	5
GA	10.9±3.3	65	5	21.7±7.0	66	4
AA	11.7±2.6	25	1	21.0±8.4	26	0
One-way ANOVA	p=0.55			p=0.28		
Two-way ANOVA (adjusted for CNV)	p=0.48			p=0.29		
Two-way ANOVA (adjusted for CNV-pooled)	p=0.44			p=0.33		

One sample missing for the genotyping from technical reasons.

B) CNV	**Age at onset (years) (mean±SD)**	**Estimate (95% CI) (years)***	**N**	**Missing**	**Y-BOCS (score) (mean±SD)**	**Estimate (95% CI) (score)***	**N**	**Missing**
Normal/High	11.2±3.0		120	7	21.7±7.6		119	8
Low	8.8±4.8	-2.5 (-0.2; -4.8)	8	0	30.6±3.7	8.7 (2.9; 14.5)	7	1
One-way ANOVA	p=0.036				p=0.003			
Two-way ANOVA*	p=0.031				p=0.004			

One sample missing for the CNV from technical reasons.

*Estimates and confidence intervals (CIs) are adjusted for SNP

Table 2: Frequencies of SNP rs6311 genotypes and CNV sizes in subjects with OCD and healthy controls

Genotype (rs6311)	**Controls N (frequency %)**	**OCD N (frequency %)**
GG	54 (50.9)	39 (28.9)
GA	35 (33.0)	70 (51.9)
AA	17 (16.0)	26 (19.3)
Fisher's exact test		p=0.002
Armitage's test for trend		p=0.007
Two-sided asymptotic (adjusted for CNV-pooled)		p=0.005
CNV	**Controls N (frequency %)**	**OCD N (frequency %)**
Normal/High	105 (99.1)	127 (94.1)
Low	1 (0.9)	8 (5.9)
Fisher's exact test		p=0.082
Two-sided asymptotic (adjusted for SNP)		p=0.020

One sample missing for the genotyping and one for CNV from technical reasons.